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Research Article

RAPD and ISSR Polymorphism in the Medicinal Plants: *Ocimum sanctum, O. basilicum* and *O. gratissimum*

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ABSTRACT

Ocimum is used in a variety of food industry and herbal drug industry. It is an important constituent of various traditional medicines. Its essential oil content gives it a unique flavour. Its popular in curing various human diseases like malaria, colic, vomiting, common cold, cough and skin problems. More than 60 varieties of *Ocimum* with a variety of leaf colour from green, to red to violet. Its important to analyze the genetic basis of this variety. In our study, we have studied four species of *Ocimum: O. sanctum, O. basilicum, O. gratissimum* and *O. americanum* taken from different geographical regions. These species differ with each other in oil contents and secondary metabolites, like: linalool, estragole, and cineole. Very few studies have been done to study the genetic variation among these species. We report a preliminary study on estimation and analysis of genetic variation through random amplification of polymorphic DNA (RAPD) and intersimple sequence repeat polymorphism (ISSR). The RAPD results showed amplified fragments in the range of 2500 to 250 bp., 60 % of them were polymorphic and the others monomorphic. We have got certain species specific bands for *Ocimum sanctum* which can be utilized for authentication of the species. Among the ten ISSR primers tested, we have got bands in the size range of 250 bp to 1 Kb., and the % polymorphism among the three species is found to be around 40%. Our results showed that both RAPD and ISSR techniques are sensitive, precise and efficient tool for genomic analysis in the species' of *Ocimum.* Such kind of studies are very useful to put *Ocimum* species in appropriate taxonomic groups. DNA based markers like ISSR are more useful than the traditional morphological and biochemical markers.

Keywords: Medicinal plant, authentication, molecular marker, RAPD, ISSR, DNA Fingerprinting

INTRODUCTION

Ocimum (Lamiaceae) is consists of aromatic herbs, undershrubs or shrubs. It is widely distributed in the tropical, subtropical and warm temperate regions of the world[1]. Nine species of *Ocimum* belong to India[2-4]. The essential oils of *Ocimum* are highly valuable in the perfumery and cosmetic industries. Its constituents are linalool, linalyl acetate, geraniol, citral, camphor, eugenol, methyl eugenol, methyl chavicol, methyl cinnamate, thymol, safrol etc. These constituents vary from species to species[4], which effect the yield and quality of essential oils[5-7]. Certain authors have reported genetic basis of this variety of essential oils. Various DNA based studies have confirmed high level of intra- and inter-specific genetic diversity in the genus *Ocimum*. However, morphological studies failed to decipher the diversity present in the genus. Morphologically similar species possess different composition of essential oil[2]. Therefore, it's imperative to study the genetic basis of the diversity of *Ocimum*. The usefulness of DNA based markers lies in their abundance, high polymorphism and freedom from environmental influences[7-10]. The markers used till now for understanding genetic diversity in *Ocimum* are AFLP (amplified fragment length polymorphism)[6,8], random amplified polymorphic DNA (RAPD)[7,9,11] and inter-simple sequence repeat(ISSR)[12]. If we especially discuss the case of RAPD markers, they amplify products of anonymous DNA sequences using single, short and arbitrary oligonucleotide primers. In many areas of science and Technology, because of its simplicity RAPD has found a wide range of applications as it does not require prior knowledge of a DNA sequence[13-15]. The main contribution of RAPD markers are majorly utilized in assessing diversity within plant populations[16,17]. In addition to it, these markers have their application for constructing linkage maps and for tracking parental origins of hybrid species[18]. The efficiency in developing large number of DNA markers in a short time with low expenditure as it requires less sophisticated equipment has made the RAPD technique to have an edge over other markers and thus made it to be valuable[19]. RAPD markers have been utilized for authentication of medicinal plants as well[13,14]. The advantages of this technique are: DNA sample is sufficient in a small amount for analysis. The factor of being useful in small amounts and not being tissue specific, which are limited in supply. As the genetic markers are not tissue specific,

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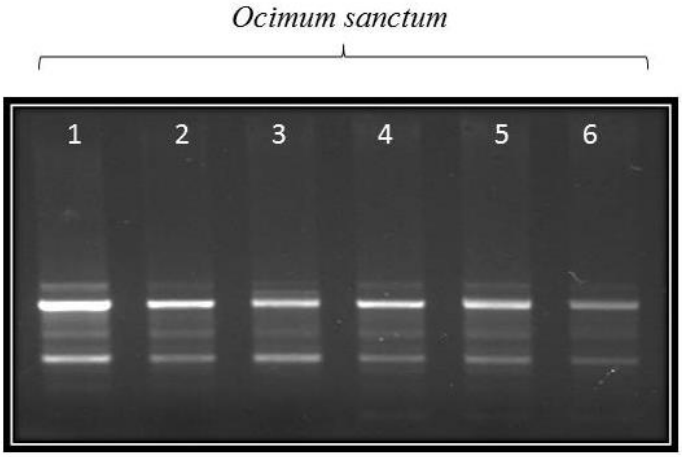

Figure 1: Banding pattern of *Ocimum sanctum* samples using RAPD primer A 1: Lane 1, 2, 3, 4, 5, and 6 contains 5 amplified bands each of sizes 2000bp, 1000bp, 750bp, 500bp and 250bp respectively.

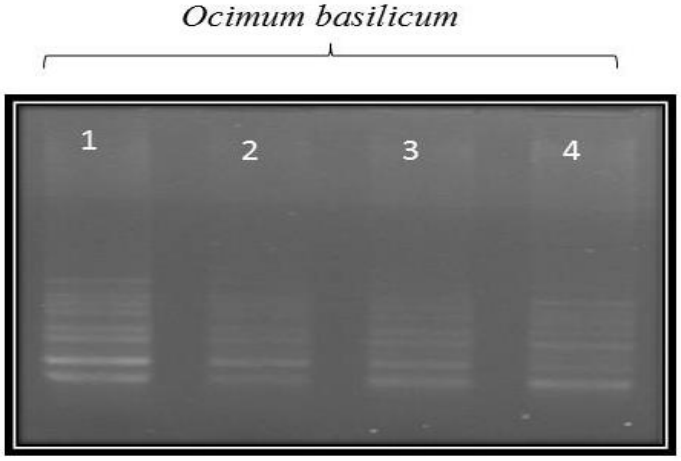

Figure 2: Banding pattern of *Ocimum basilicum* samples using RAPD primer G 2: Lane 1, 2, 3 and 4 contains 8 amplified bands of sizes 2500bp, 2000bp, 1000bp, 900bp, 750bp, 600bp, 500bp and 250bp respectively.

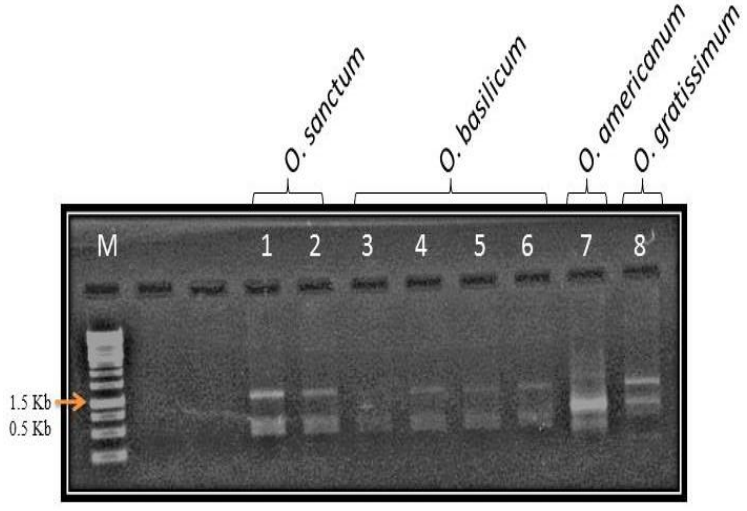

Figure 3: Banding pattern of *Ocimum sanctum, Ocimum basilicum, Ocimum americanum* and *Ocimum gratissimum* samples using RAPD primer A 5: Lane 1, 2 each contains 5 amplified bands of *Ocimum sanctum* samples of sizes 2000bp, 1000bp, 750bp, 500bp and 250bp respectively; Lane 3, 4, 5 and 6 contains 4 amplified bands of *Ocimum basilicum* samples of sizes 2500bp, 1000bp, 750bp and 250bp respectively; Lane 7 contains 3 amplified bands of *Ocimum americanum* of sizes 1500bp, 500bp and 250bp; Lane 8 contains 4 amplified bands of *Ocimum gratissimum* of sizes 1000bp, 900bp, 750bp and 250bp.

so they can be detected at any stage of development. Inter-simple sequence repeat (ISSR) is a PCR-based method developed by Zietkiewicz et al.[20]. It utilizes repeat-anchored primers to amplify DNA sequences between two inverted SSRs. Because of high annealing temperature of ISSR primers, this technique is highly reliable, reproducible besides being cost-effective. ISSRs have been successfully used to estimate the extent of genetic diversity at inter- and intra-specific level in a wide range of medicinal plant species[21,22]. None of the DNA based markers have been utilized for authentication of the genus *Ocimum*. In the present study, we have utilized for the first time RAPD and ISSR markers for genetic diversity studies and authentication of *Ocimum spp. viz., sanctum, basilicum, americanum and gratissimum.*

MATERIALS AND METHODS

Source

Ocimum species var. Shyama and Basil were collected from the herbal gardens of Amity University, Noida and Jamia Hamdard (New Delhi); *Ocimum gratissimum* sample were collected from the gardens of National Botanical Research Institute (NBRI), Lucknow.

DNA Extraction

Young leaf tissue was excised from the plants, washed thoroughly with distilled water and wiped off with clean tissue paper to remove surface water completely and lyophilized. Genomic DNA was extracted from using 100 mg of lyophilized leaf material our modified protocol[23] and stored at -20° C till further use. DNA was quantified using agarose gel electrophoresis and 1 Kb DNA Ladder served as standard. Qualitative and quantitative assessment was done by 0.8% agarose gel electrophoresis.

RAPD Marker Analysis

A set of 10 random primers (Imperial Life Sciences, New Delhi) were used for initial screening. Randomly amplified polymorphic DNA assay was carried out in 15 µl reaction volume containing 50 ng DNA, 5 mM $MgCl_2$, 2 mM each of dNTP, 10 µM random decamers, 0.6 U *Taq* polymerase (Genei). Amplification was performed with thermal cycler (Eppendorf Germany) using the cycling parameters of Sarwat et al.[24] One cycle of 94ºC for 2 min, 37 ºC for 2 min, 72 ºC for 2 min followed by 30 cycles of 94 ºC for 1 min, 37 ºC for 1 min, 72 ºC for 1 min. The last cycle was followed by 10 min extension at 72 ºC. The amplified products were resolved in 1.2% agarose gel (0.5 X TBE), stained with ethidium bromide (10 µg/ml) and photographed under UV light.

ISSR Marker Analysis

For ISSR analysis, a set of 20 primers were synthesized from commercial source. These primers were tested for their suitability for the *Ocimum* species studied. Ten primers were found suitable. 20 ng of template DNA was subjected to amplification with 7.5 µM of 3`-anchored microsatellite primers, other components of the reaction mixture include 5 mM $MgCl_2$, 2 mM each of dNTP and 0.6 U *Taq* polymerase (Genei). Electrophoresis was performed on 1.2% agarose gel. Amplification was performed with thermal cycler (Eppendorf Germany) using the cycling parameters of Sarwat et al.[24] The annealing temperatures of the cycling parameter were readjusted for each microsatellite primers according to their calculated melting temperature (Tm) based on the sequence composition [Tm = 4 (G + C) + 2 (A + T) - 3 C]. The amplified products were resolved in 1.2% agarose gel (0.5 X TBE), stained with ethidium bromide (10 µg/ml) and photographed under UV light.

Statistical Analysis

The positions of the amplified bands were compared for each accession and primer combination. The non reproducibleand ambiguous bands were eliminated. The bands were scored as 1 and 0 based on the basis of presence and absence, respectively. Sizes of amplified bands were estimated using Gel Pro analyzer software. This gel was evaluated for final (Monomorphic and Polymorphic) bands and % polymorphism was found out by calculating the number of polymorphic bands divided by the total number of bands.

RESULTS

The DNA obtained was of very high quality 50 ng to 100ng per µl. The quality was checked both electrophoretically and spectrophotometrically confirmed its high quality which can be utilized for PCR based analyses. Out of those ten, 4 primers (A1, A4, A5 and G2) yielded good results. The intraspecific diversity analyses among different accessions of *Ocimum sanctum* showed 100 % monomorphic bands (Figure 1). Thus, our intraspecific study of *Ocimum sanctum* tells us that though the samples were taken from different places, they were morphologically and genetically similar, depicting that there is not much genetic diversity in *Ocimum sanctum* plants. Similar results were obtained by intraspecific study of different accessions of *Ocimum basilicum* taken from different regions (Figure 2). In this experiment, we found out species- specific bands to differentiate between *Ocimum sanctum* and *Ocimum basilicum*, which can be used to identify specific *Ocimum* species, amongst these two, according to the requirement. Along with this, we also found out that the bands for different plants of *Ocimum sanctum* were mostly of similar size, which tells us that these were not genetically diverse. Interspecific study using RAPD primers A 4, A 5 show that bands of size of 2500 bp and 1500 bp are species- specific markers for *Ocimum basilicum* and *Ocimum americanum as* shown in Figure 1 and 2. In case of Interspecies analysis, % polymorphism was found out to be 60% using RAPD primer A 4 which showed that there exists a considerable discrimination between *Ocimum sanctum* and *Ocimum basilicum* samples which were taken from different regions as shown in Table 1 & 2, Figure 3. Results of ISSR Analyses tallies with our study on RAPD markers, depicting that ISSR confirms the presence of genetic diversity amongst the three *Ocimum* species (i.e. interspecific genetic diversity), the % polymorphism with S1 was found to be 41.66% (Fig. 4, Table 3) while with S8 was found to be 38.06% (Fig. 5, Table 3), giving a cumulative polymorphism as 40%.

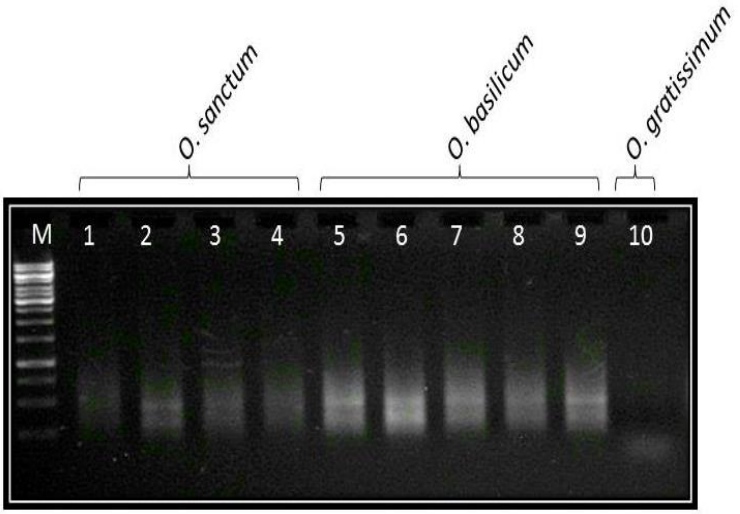

Figure 4: Banding pattern using ISSR primer S 1 from Lane 1 to Lane 10; Lane 1, 2, 3, 4 contains 2 amplified bands of *Ocimum sanctum* of sizes 500bp and 250bp; Lane 5, 6, 7, 8 and 9 contains 3 amplified bands of *Ocimum basilicum* samples each of sizes 1000bp, 500bp and 250bp; Lane 10 contains no amplified bands of *Ocimum gratissimum* .

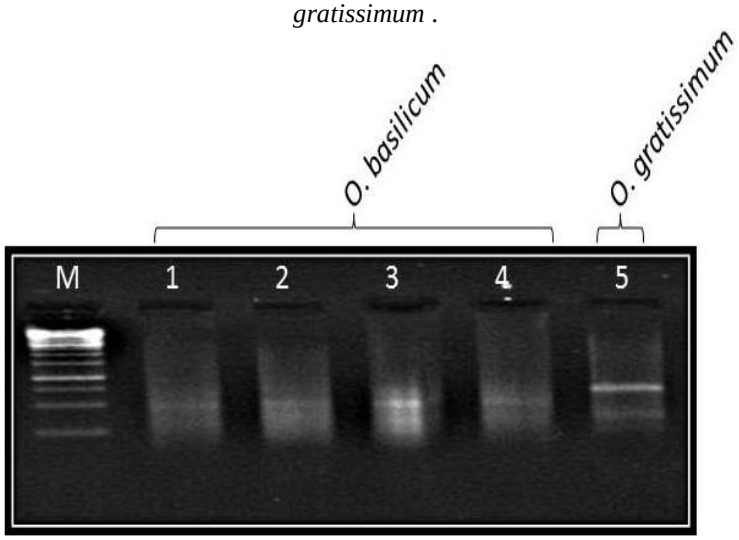

Figure 5: Banding pattern using ISSR primer S 8 for *Ocimum basilicum* and *Ocimum gratissimum* samples; Lane 1, 2, 3 and 4 contains 2 amplified bands of *Ocimum basilicum* each of sizes 500 bp and 300bp; Lane 5 contains 3 amplified bands of *Ocimum gratissimum* samples of sizes 750bp, 500bp and 250bp.

Table 1: Number of monomorphic and polymorphic bands produced with each of these primers

S no.	No. Of bands	Monomorphic bands	Polymorphic bands	% polymorphism
A 1	35	35	0	0
A 4	60	24	36	36/60 = 60%
A 5	29	22	7	7/22 = 31.81%
G 2	24	24	0	0

Through ISSR analysis we have got bands in the size range of 250 bp to 1 Kb (Table 4).

DISCUSSIONS

RAPD can give results on a number of loci, higher than any morphological or biochemical markers[25]. It is easy to use these markers because they are less time consuming and less labour intensive besides being economical[26]. These markers are shown to detect high polymorphism than RFLP and AFLP markers[27,28]. The previous studies based on the morphological, cytological and oil characterstics grouped eleven species of *Ocimum* into two sections[29,30]. *O. basilicum* was grouped with *O. americanum* and *O. tenuiflorum* was grouped with *O. gratissimum*. Molecular marker studies unravel the existing genetic diversity among these species and gave more objective classifications. In *Ocimum* spp. RAPD have been utilized by various scientists for the evaluation of genetic diversity, interrelationship and phylogeny[31], It may be due to the possibility that most of the genetic loci screened with RAPD markers were polymorphic w

Table 2: Band sizes of the products obtained from RAPD primers

PRIMER A 1			PRIMER A 4			PRIMER G 2			PRIMER A 5		
LANE	BAND	SIZE OF BAND(IN Kb)	LANE	BAND	SIZE OF BAND (In kb)	LANE	BAND	SIZE OF BAND (In kb)	LANE	BAND	SIZE OF BAND (In kb)
2	1	2000	8	1	2500	3	1	2500	4	1	2000
	2	1000		2	2000		2	2000		2	1000
	3	750		3	1500		3	1000		3	750
	4	500		4	1000		4	900		4	500
	5	250		5	900		5	750		5	250
3	1	2000		6	750		6	600	5	1	2000
	2	1000		7	600		7	500		2	1000
	3	750		8	500		8	250		3	750
	4	500		9	250	4	1	2500		4	500
	5	250	9	1	2500		2	2000		5	250
4	1	2000		2	2000		3	1000	7	1	2500
	2	1000		3	1500		4	900		2	1000
	3	750		4	1000		5	750		3	750
	4	500		5	900		6	600		4	250
	5	250		6	750		7	500	8	1	2500
5	1	2000		7	600		8	250		2	1000
	2	1000		8	500	5	1	2500		3	750
	3	750		9	250		2	2000		4	250
	4	500	10	1	2500		3	1000	9	1	2500
	5	250		2	2000		4	900		2	1000
6	1	2000		3	1500		5	750		3	750
	2	1000		4	1000		6	600		4	250
	3	750		5	900		7	500	10	1	1500
	4	500		6	750		8	250		2	500
	5	250		7	600	6	1	2500		3	250
7	1	2000		8	500		2	2000	11	1	1000
	2	1000		9	250		3	1000		2	900
	3	750	11	1	2500		4	900		3	750
	4	500		2	2000		5	750		4	250
	5	250		3	1500		6	600			
				4	1000		7	500			
				5	900		8	250			
				6	750						
				7	600						
				8	500						
				9	250						
			15	1	2500						
				2	2000						
				3	1000						
				4	900						
				5	750						
				6	600						
				7	500						
				8	250						
			16	1	2500						
				2	2000						
				3	1000						
				4	900						
				5	750						
				6	600						
				7	500						
				8	250						
			17	1	2500						

2	2000
3	1000
4	900
5	750
6	600
7	500
8	250

Table 3: No of polymorphic and monomorphic bands produced by each of the ISSR primers

ISSR primer	No. Of bands	Polymorphic bands	Monomorphic bands	% polymorphism
S 1	17	5	12	5/17= 41.66%
S 8	27	8	21	8/21= 38.09%

Table 4: Band sizes of products obtained from ISSR primers

Primer s 1			Primer s 8		
Lane	Band	Size of band	Lane	Band	Size of band
3	1	500	12	1	1000
	2	250		2	750
6	1	1000		3	500
	2	500		4	250
	3	250	13	1	1000
7	1	1000		2	750
	2	500		3	500
	3	250		4	250
8	1	1000	14	1	1000
	2	500		2	750
	3	250		3	500
9	1	1000		4	250
	2	500	15	1	1000
	3	250		2	750
10	1	750		3	500
	2	500		4	250
	3	250	16	1	500
				2	300
			17	1	500
				2	300
			18	1	500
				2	300
			19	1	500
				2	300
			20	1	750
				2	500
				3	250

respect to one or more species. High level of genetic dissimilarity (up to 80%) was observed between *Ocimum* species, while the high intraspecific genetic divergence in *O.basilicum* and *O. tenuiflorum* germplasm. Through RAPD markers high degree of polymorphism (98.28%) have been revealed among the 32 accessions consisting of five *Ocimum* species[32]. Singh et al.[9] used RAPD markers to observe genetic relationships among thirty germplasm accessions from five *Ocimum* species. Very high degree of polymorphism (98.20%) have been recorded in this study. The intraspecific diversity is reported to be much lower (44.83%) as shown by Ibrahim et al.[33] in the three varieties of *Ocimum basilicum*. According to Lal et al.[32] *O. basillicum* and *O. gratissimum* were not so similar so that they can be included in one group. Contrarily, Harisaranraj et al.[11] showed *O. basillicum* to be more similar to *O. gratissimum* than *O. americanum*. Also *O. basillicum* and *O. gratissimum* fall in one group in dendogram. These kind of divergent data necessitates the use of more than one marker system to analyse genetic diversity. Another useful marker employed for characterization of genetic diversity is ISSR. Some studies[34,35] have considered ISSR to be a better tool than RAPD for phylogenetic studies. ISSR primers are considered to be more informative than RAPD[24,36]. Whereas, others showed equal efficiency of both RAPD and ISSR markers[37]. They reported that both markers are equally useful for differentiating the closely related cultivars of *Catharanthus roseus*. Another study by Lalhruaitluanga and Prasad[38] on *Melocanna baccifera* Roxb. showed comparative analyses of RAPD and ISSR markers for genetic diversity. High level of polymorphism was detected 98.02% (through RAPD) and 84.1% (through ISSR). The combined analyses of RAPD, ISSR and SSR revealed that *Ocimum basilicum* and *Ocimum americanum* were the most similar and *Ocimum viride* and *Ocimum americanum* are highly dissimilar[32]. Another study by Chen et al.[39] on 37 accessions of *Ocimum* belonging to 4 species (*O. basilicum, O. americanum, O. gratissimum* and *O. tenuiflorum*) also revealed high level of genetic polymorphism among the species. ISSR exibited the highest polymorphism (97%) followed by RAPD (95%) and SRAP (93%). This study also shows that a combination of markers (ISSR, RAPD and SRAP) is more useful as it spans different regions of the genome. Our results corroborate with the above findings, as we got high level of genetic dissimilarity between the three species of *Ocimum* studied, thus demonstrating substantial genetic variation at the level of within species and between species. This shows that the genetic base of *Ocimum* genus is quite broad.

CONCLUSIONS

The species specific markers amplified in this study can be converted into simple PCR-based sequence-characterized amplified region (SCAR) markers which will allow the screening of large number of samples and populations from different regions. All markers can be tested against other potential species. A set of highly

differentiating markers can be used as diagnostic markers to identify the raw material for herbal drugs of different species and different regions, the data of which would contribute to the authentication of herbal medicines, to avoid batch to batch variations in extraction of standard drugs and to devise strategies for forest conservation and biodiversity.

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